

## Indirect competitive immunoassay for detection of aflatoxin B<sub>1</sub> in corn and nut products using the array biosensor

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### Abstract

Because of the potential health risks of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), it is essential to monitor the level of this mycotoxin in a variety of foods. An indirect competitive immunoassay has been developed using the NRL array biosensor, offering rapid, sensitive detection and quantification of AFB<sub>1</sub> in buffer, corn and nut products. AFB<sub>1</sub>-spiked foods were extracted with methanol and Cy5-anti-AFB<sub>1</sub> added to the resulting sample. The extracted sample/antibody mix was passed over a waveguide surface patterned with immobilized AFB<sub>1</sub>. The resulting fluorescence signal decreased as the concentration of AFB<sub>1</sub> in the sample increased. The limit of detection for AFB<sub>1</sub> in buffer, 0.3 ng/ml, was found to increase to between 1.5 and 5.1 ng/g and 0.6 and 1.4 ng/g when measured in various corn and nut products, respectively.  
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### 1. Introduction

The mycotoxin aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a metabolite mainly produced by the fungi *Aspergillus flavus* and *A. parasiticus* (Bennett and Klich, 2003). The fungi grow on a number of agricultural products, under favorable conditions of temperature and pressure, prior to harvest or during storage. Aflatoxins have commonly been found to contaminate corn and corn products, peanuts and peanut products, cottonseed and tree nuts such as Brazil nuts, pecans and pistachio nuts (Stroka and Anklaam, 2002). Exposure to aflatoxins typically results from the ingestion of contaminated foodstuffs. These fungal toxins are found in animal studies to be strong hepatotoxins and potent carcinogens and are therefore considered extremely hazardous to humans (IARC, 1993; Jarvis and Miller, 2005). The European Commission set the maximum level for AFB<sub>1</sub> in foods to 2 ng/g, although new limits are likely to be established at 1 ng/g (Stroka and Anklaam, 2002). Common established methodolo-

gies for aflatoxin detection include thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC), and while these techniques have excellent sensitivities, they typically require skilled operators, extensive sample pretreatment and expensive equipment (Stroka and Anklaam, 2002; Papp et al., 2002). The goal of more recent studies has been to simplify and expedite the method of detection while attempting to maintain or improve the sensitivity. The production of antibodies to AFB<sub>1</sub> has greatly aided in this endeavor, leading to the development of a variety of antibody-based methods. Antibodies have been used to clean up samples prior to measurement by LC (Urano et al., 1993), HPLC (Garcia-Villanova et al., 2004) or capillary electrophoresis (Maragos and Greer, 1997) with a concomitant increase in assay sensitivity. Antibody-based detection methods for AFB<sub>1</sub> include standard immunoassays coupled with colorimetric (Garden and Strachan, 2001; Delmulle et al., 2005; Xiulan et al., 2005), electrochemical (Ammida et al., 2004) or surface plasmon resonance (Daly et al., 2000; Dunne et al., 2005) detection, and enhanced immunoassays such as the enzyme-linked immunosorbent assay (ELISA) (Bhattacharya et al., 1999; Pal and Dhar, 2004; Lee et al., 2004).

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The majority of these detection systems, however, lack the ability to either perform simultaneous analysis of multiple samples or simultaneous analysis of multiple target analytes. In recent years the development of array-based biosensors has offered the ability to measure multiple samples simultaneously for multiple target analytes. The NRL array biosensor typically uses patterned antibodies and sandwich immunoassays and has successfully been used for the detection of a variety of species in food matrices (Feldstein et al., 1999; Taitt et al., 2004a, 2004b; Shriver-Lake et al., 2003, 2004; Sapsford et al., 2005; Ngundi et al., 2005). In this study we develop an indirect competitive immunoassay for the low molecular weight analyte AFB<sub>1</sub> in buffer and demonstrate the efficiency of that method for measuring AFB<sub>1</sub> spiked into a variety of corn and nut products. The spiked foodstuffs were prepared to represent naturally contaminated samples in order to investigate any potential matrix effects on the immunoassay performance.

## 2. Materials and methods

### 2.1. Materials

Unless otherwise specified, chemicals were of reagent grade and used as received. Borosilicate glass slides from Daigler & Co. Inc. (Vernon Hills, IL) were used as waveguides in all the assays described. Poly(dimethyl)siloxane (PDMS), used for making the assay flow cells, was obtained from Nusil Silicone Technology (Carpinteria, CA). The 3-mercaptopropyl trimethoxysilane (MTS) and *N*-( $\gamma$ -maleimidobutyryloxy) succinimide ester (GMBS) were purchased from Fluka Chemical Co. (St. Louis, MO). EZ-Link biotin-LC-PEO-amine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and NeutrAvidin were obtained from Pierce (Rockford, IL). The biotin-SP-conjugated rabbit anti-chicken IgY and Cy5-chicken IgY, used as positive controls, were purchased from Jackson ImmunoResearch (West Grove, PA). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) was purchased from Sigma (St. Louis, MO). Fluorescent labeling of the antibodies was achieved using Cy5 bisfunctional dye from Amersham Biosciences (Arlington Heights, IL). Bovine serum albumin (BSA), gelatin, potassium hydroxide, sodium chloride, sodium phosphate mono- and di-basic, polyoxyethylenesorbitan monolaurate (Tween 20), dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), poly(ethylene glycol)-average weight 8000-(PEG), poly(vinylpyrrolidone) (PVP) and toluene were supplied by Sigma-Aldrich. Absolute ethanol was obtained from Warner-Graham Co. (Cockeysville, MD). All food was purchased from local grocery stores. A Waring two-speed commercial blender equipped with a mini-sample container (37–110 ml), used for some of the food preparation, and isopropyl alcohol were purchased from Fisher Scientific (Pittsburgh, PA).

### 2.2. Preparation of biotin-AFB<sub>1</sub>

The synthesis of biotin-AFB<sub>1</sub> was a two-step process in which an oxime intermediate of the AFB<sub>1</sub> was first prepared. Into a 50 ml flask was placed 31 mg of carboxymethyl hydroxy-

lamine hemihydrochloride (from Aldrich) followed by 20 mg of AFB<sub>1</sub>, dissolved in 1:4:1 pyridine: methanol: water. The reaction was refluxed for 2 h and cooled, and the solvents were removed by rotary evaporation. The resulting AFB<sub>1</sub>-(*O*-carboxy methyl) oxime intermediate was characterized by positive ion LC mass spectrometry equipped with a nano spray source.

In the second part of the synthesis, 15 mg NHS and 30 mg EDC were added to a flask which was then closed with a septum. A solution of 24.6 mg AFB<sub>1</sub>-(*O*-carboxy methyl) oxime intermediate dissolved in 3 ml of DMF was then added to the flask. Following 1 h of stirring, a solution of 50 mg of biotin-LC-PEO-amine in 2 ml 0.05 M carbonate-bicarbonate buffer (pH 9.5) was added to the flask and stirred for 24 h at 4 °C. The reaction mixture was then transferred to a 500 MW dialysis bag and dialyzed for several days against 1 l PBS with at least three changes of buffer. The resulting biotin-AFB<sub>1</sub> product was characterized by positive ion LC mass spectrometry and UV-vis spectroscopy and then stored in a vial at 4 °C.

### 2.3. Preparation of monoclonal mouse antibodies against AFB<sub>1</sub>

The antibodies were prepared at the USDA-NCAUR (Peoria, IL) as described. The carboxymethyloxime of AFB<sub>1</sub> (AFB<sub>1</sub>-CMO) was prepared according to the method of Chu et al. (1977). The AFB<sub>1</sub>-CMO was coupled to keyhole limpet hemocyanin (KLH) with 1,1'-carbonyldiimidazole using a method similar to that of Xiao et al. (1995) for coupling AFB<sub>1</sub>-CMO to ovalbumin. Aflatoxin B<sub>3</sub> (AFB<sub>3</sub>) was also linked with ovalbumin to produce a second conjugate (AFB<sub>3</sub>-OVA). Five Balb/C mice were immunized with AFB<sub>1</sub>-KLH at the University of Illinois Immunological Resource Center (Urbana, IL). Sera were screened at the USDA-NCAUR for activity in competitive ELISAs with AFB<sub>3</sub>-OVA as the solid phase test antigen and goat anti-mouse-HRP as the secondary antibody. Lymphocytes from a selected mouse were fused with the Balb/C non-immunoglobulin secreting mouse myeloma cell line SP2/0-Ag14. Cultures positive for aflatoxin-binding antibodies were cloned, expanded and screened for activity. The resulting cell line, P2G8-2-D2, produced an anti-aflatoxin antibody of isotype IgG1, with a lambda light chain.

Upon arrival at the NRL, the antibodies were further purified using MEP HyperCel<sup>®</sup> hydrophobic charge induction chromatography (Pall Corporation, East Hills, NY) to ensure complete removal of any BSA or other proteins that could otherwise interfere with labeling procedures.

### 2.4. Preparation of labeled antibodies

Cy5-labeling of the monoclonal mouse antibody to AFB<sub>1</sub> was performed according to the manufacturer's instructions. Labeled antibodies were separated from unincorporated dye using size exclusion chromatography (BioGel P10). The protein-to-dye ratio, determined using UV-vis spectroscopy, was calculated to be 1:2.

## 2.5. Patterning biotinylated capture species using PDMS flow cells

Microscope slides, used as waveguides, were cleaned by immersion in a 10% (w/v) KOH in 2-propanol for 30 min at room temperature, followed by rinsing with deionized water and drying with a nitrogen stream. The slides were immediately immersed in a toluene solution containing 2% MTS for 1 h under nitrogen (Bhatia et al., 1989). The silanized slides were then rinsed with toluene, dried with nitrogen and immediately immersed in 1 mM GMBS in absolute ethanol for 30 min at room temperature. The slides were rinsed with water and incubated in 25  $\mu\text{g/ml}$  NeutrAvidin in phosphate buffered saline (PBS) overnight at 4 °C. They were then washed in PBS and either used immediately for patterning or stored in PBS at 4 °C until required. Patterning of the biotinylated AFB<sub>1</sub> was carried out using a 6- or 12-channel patterning PDMS flow cell clamped onto the NeutrAvidin functionalized waveguide surface (Taitt et al., 2004b). Biotin-conjugated AFB<sub>1</sub> (2  $\mu\text{g/ml}$ : a concentration determined after initial optimization experiments, as described in Section 3.1) in PBS + 0.05% Tween (PBST) was introduced into the channels of the flow cell. Biotinylated anti-chicken IgY (10  $\mu\text{g/ml}$  in PBST) and biotin-labeled fumonisin B (FB; 2  $\mu\text{g/ml}$  in PBST) were introduced into the remaining channels, for use as positive and negative controls, respectively. The slides were then incubated overnight at 4 °C. After the flow cell channels were rinsed with 1 ml PBST, the slide was removed from the PDMS template and placed in PBST blocking solution containing 1% gelatin. After 30 min, the slides were rinsed with Milli-Q water and assembled in a 6- or 12-channel assay PDMS flow cell.

## 2.6. Food preparation and spiking

To prepare representative samples of naturally contaminated food stuffs for analysis, various corn and nut products were spiked and incubated overnight with AFB<sub>1</sub>. Cornflakes and cornmeal were blended to a fine texture using a commercial blender. Unpopped popcorn, peanuts, peanut butter and pecan nuts were used as received. AFB<sub>1</sub> spike solutions were prepared in 100% methanol at concentrations ranging from 0 to 80 ng/ml and 125  $\mu\text{l}$  of the spike solution was added per 0.5 g of food previously aliquotted and weighed. The samples were vortexed and the solvent allowed to evaporate overnight from the open sample vials in the fume hood.

## 2.7. Extraction of AFB<sub>1</sub> from food samples

Each spiked sample aliquot was then extracted with 1–1.5 ml of 75% methanol/water (v/v), first by vortexing and then by shaking on a horizontal shaker for 2 h at room temperature. In the case of the cornflake, cornmeal and peanut butter samples, the spiked aliquots were then centrifuged at 3000 rpm for 5 min and the resulting supernatant collected. For the popcorn, peanuts and pecan samples, liquid was removed from the food samples using a Pasteur pipet. An aliquot (0.33 ml) of each sample was diluted three-fold with PBST containing BSA, Cy5-anti-AFB<sub>1</sub> and Cy5-chicken IgG such that the final concentrations were

0.66 mg/ml, 2  $\mu\text{g/ml}$  and 50 ng/ml, respectively. In addition PEG was added to the nut extracts to a final concentration of 0.66%. The extract/antibody mix was incubated for 20 min at room temperature before analysis.

## 2.8. Indirect competitive immunoassays

The NeutrAvidin slides patterned with the biotinylated AFB<sub>1</sub> were assembled with assay PDMS flow cells, such that the flow channels were orientated perpendicular to the strips of immobilized biotinylated molecules. Each channel was hooked up to an ISMATEC® multi-channel pump (Cole-Parmer Instruments Company, Vernon Hills, IL) at one end (outlet) and syringe barrels (1 ml) were then attached at the opposite end (inlet). The PDMS channels were first washed with 1 ml of PBSTB (1 mg/ml BSA) at 0.5 ml/min, to check for any leaks in the PDMS flow cell. Next, 0.8 ml of spiked food sample, containing “free” AFB<sub>1</sub> and the Cy5-labeled monoclonal mouse anti-AFB<sub>1</sub>, was applied to each channel at a flow rate of 0.15 ml/min. The channels were then washed with 1 ml of PBSTB at 0.5 ml/min. The PDMS flow cell was removed, and the slide was washed with Milli-Q water, dried with nitrogen and imaged on the array biosensor.

## 2.9. Slide imaging and data analysis

The slide was imaged using a Peltier-cooled CCD camera, which has previously been described (Feldstein et al., 1999), see Fig. 1. Briefly, evanescent wave excitation of the surface-bound fluorescent species was achieved using a 635 nm, 12 mW diode laser (Lasermix, Rochester, NY). Light was launched into the end of the waveguide at an appropriate angle through a 1 cm focal length lens equipped with a line generator. The fluorescence emission was monitored at right angles to the planar surface of the waveguide. A two-dimensional graded index of refraction (GRIN) lens array (Nippon Sheetglass, Summerset, NJ) was used to image the fluorescent pattern onto the Peltier-cooled CCD camera (Spectra Source, Teleris, Westlake Village, CA) (Golden, 1998). Long-pass (Schott OG-0665, Schott Glass, Duryea, PA) and band-pass filters (Corion S40-670-S, Franklin,

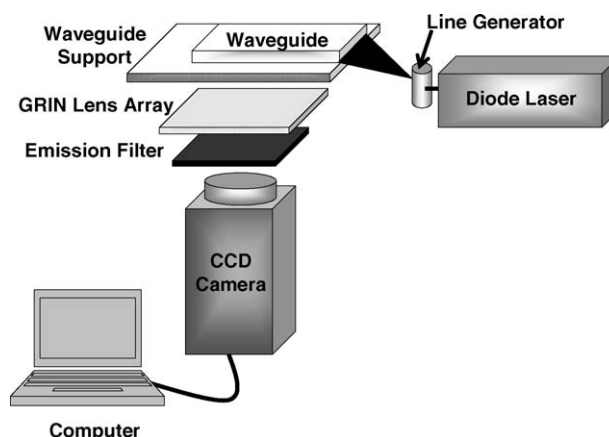


Fig. 1. Schematic of the NRL array biosensor used for the current study.

MA) were mounted on the device scaffolding to eliminate excitation and scattered light prior to CCD imaging.

Data were acquired in the form of digital image files in flexible image transport system (FITS) format. To analyze the images, a custom software application was written in LabWindows/CVI (National Instruments). The program creates a mask consisting of data squares (enclosing the areas where the capture antibody is patterned) and background rectangles, which are located on either side of each data square. The average background value is subtracted from the average data square value, and net intensity value is calculated and imported into a Microsoft Excel file for data analysis. The indirect competitive immunoassay format utilizes the competition between free AFB<sub>1</sub> in solution and immobilized AFB<sub>1</sub> for binding of the dye-labeled antibody to quantify the mycotoxin in the sample. Since these competitive assays result in a decrease in fluorescent signal with increasing concentration of free AFB<sub>1</sub> in the sample, the intensity values were plotted as the percent inhibition of the fluorescent signal as given in Eq. (1)

$$\% \text{ inhibition} = \left[ 1 - \frac{\text{fluorescence A}}{\text{fluorescence B}} \right] \times 100 \quad (1)$$

where fluorescence A is the fluorescence obtained using the tracer in the presence of solution AFB<sub>1</sub> and fluorescence B is the fluorescence obtained by the tracer alone (both in the matrix of interest). Limits of detection (LODs) were calculated as the lowest tested concentrations at which the % inhibition was at least 3 standard deviations above the mean % inhibition of the tracer alone in the matrix of interest, in the absence of exogenously (spiked) added toxin.

### 2.10. Safety considerations

Due to the toxic nature of AFB<sub>1</sub> all solutions were handled by personal wearing appropriate personal protective gear (goggles, lab coats and gloves). All surfaces, glassware and other containers that were used or came into contact with AFB<sub>1</sub> were treated with 20% bleach before disposal. Food samples spiked with AFB<sub>1</sub> were clearly labeled as a biohazard, kept in a separate fume hood, and placed in biohazard bags for disposal by incineration.

## 3. Results and discussion

The NRL array biosensor has successfully been used for the rapid detection and quantification of a number target analytes in a variety of complex samples matrices (Taitt et al., 2004a, 2004b; Shriver-Lake et al., 2003, 2004; Sapsford et al., 2005). Most commonly, detection schemes involve the use of sandwich immunoassays. This immunoassay format is suitable for analyte species large enough to contain separate recognition sites for both the capture and tracer antibodies; however, mycotoxins and other small molecules are too small to meet this requirement. We have previously demonstrated that small analytes such as TNT (Sapsford et al., 2002) and the mycotoxin ochratoxin A (OTA) (Ngundi et al., 2005) can be detected using either displacement and/or competitive immunoassays in various arrange-

ments. Based on the indirect competitive immunoassay format developed for OTA (Ngundi et al., 2005), biotinylated AFB<sub>1</sub> was patterned on to the waveguide surface where it competed with AFB<sub>1</sub> free in solution for binding sites on the Cy5-labeled anti-AFB<sub>1</sub> also in solution. Unlike the sandwich immunoassay, the indirect competitive immunoassay format produced a decreasing fluorescence signal with increasing concentration of AFB<sub>1</sub> in solution.

### 3.1. AFB<sub>1</sub> indirect competitive immunoassay; optimization in buffer

Initially, different concentrations of immobilized, biotinylated AFB<sub>1</sub> were exposed to various concentrations of Cy5-labeled anti-AFB<sub>1</sub> to determine the optimal concentration of both that would give the best assay sensitivity. All further indirect competitive assays were then performed using these optimized conditions. Various solvent systems have been used for extraction of AFB<sub>1</sub> from foodstuffs, including, chloroform, methanol and acetonitrile (Papp et al., 2002). For this study we chose methanol extraction due to its relative ease of use and disposal. To test compatibility of the current AFB<sub>1</sub> immunoassay with methanol, assays were performed in PBSTB containing various methanol concentrations ranging from 0 to 25% (data not shown). Results demonstrated that 25% methanol did not produce final fluorescence signals consistently higher or lower than the 0% methanol and could therefore safely be used in the assay without detriment to the tracer antibody. Fig. 2A shows the CCD image of a slide exposed to competitive assays of unlabeled AFB<sub>1</sub> in buffer plus 25% methanol using the optimized conditions. The patterned slide contained both positive control lanes (Rb-anti-chicken IgG) and negative control lanes, in which a biotin-labeled fumonisin (FB) was immobilized on the NeutrAvidin surface. It is clear from the CCD image that as the concentration of unlabeled AFB<sub>1</sub> in solution increases, the final fluorescence intensity decreases. Binding of the Cy5-labeled anti-AFB<sub>1</sub> antibody to immobilized biotin-FB was not significantly above background levels ( $P > 0.2$ , unpaired  $t$ -test). The net intensities obtained from the CCD image were plotted as a standard dose-response curve using the % inhibition, determined using Eq. (1), as shown in Fig. 2B. The % inhibition curve for AFB<sub>1</sub> in buffer + 25% methanol could be fitted to a sigmoidal four-parameter equation,  $Y = -21.9 + \{124.1/[1 + \exp(-(x - 0.21)/0.14)]\}$ , with  $R^2 = 0.998$ . The LOD was  $0.28 \text{ ng/ml} \pm 0.07 \text{ ng/ml}$  (average measured LOD for four slides). Unlike OTA assays, optimized for the same biosensor (Ngundi et al., 2005), the dynamic range for the AFB<sub>1</sub> indirect competitive immunoassay was very sharp; typically extending over a  $\leq 1.0 \text{ ng/ml}$  change in concentration.

### 3.2. AFB<sub>1</sub> indirect competitive immunoassay; corn and nut products

Various corn and nut products were spiked with different concentrations of AFB<sub>1</sub>, and the mycotoxin extracted using 75% methanol in water. The extracts were then diluted in PBSTB



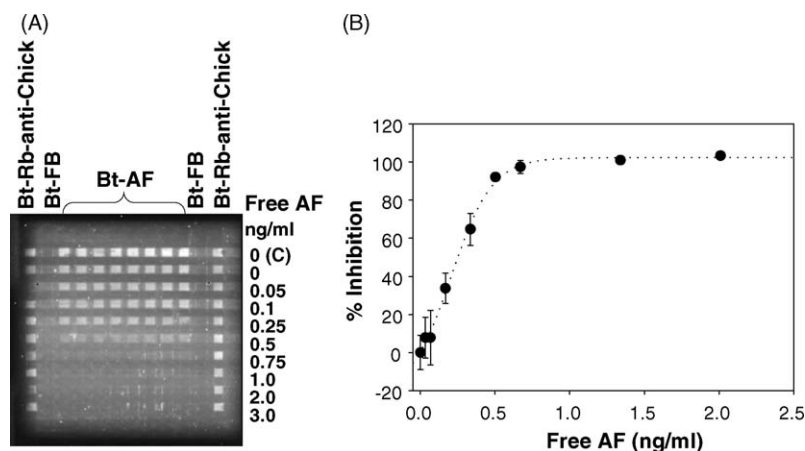


Fig. 2. (A) Competitive immunoassays for AFB<sub>1</sub> in PBSTB + 25% methanol. The CCD image showing positive and negative control columns of Rb anti-chicken IgG and Bt-FB, respectively, as well as AFB<sub>1</sub>-specific columns, was collected after a 6 min exposure of the waveguide to the sample followed by a buffer wash. A positive control (C) containing Cy5 anti-AFB<sub>1</sub> only in PBSTB was run as part of the assay in the first lane. (B) Dose-response curve for AFB<sub>1</sub> in PBSTB + 25% methanol. Values for fluorescence background were calculated from the CCD image and plotted as the mean % inhibition of the Cy5 anti-AFB<sub>1</sub> versus the concentration of AFB<sub>1</sub> in the buffer. Error bars are the standard deviation of the mean,  $n = 8$ .

containing the tracer antibody, to give a final concentration of 25% methanol and analyzed via indirect competitive assays using biotinylated AFB<sub>1</sub>-patterned waveguides. Two negative controls, without AFB<sub>1</sub>; a buffer control (PBSTB, Cy5-anti-AFB<sub>1</sub>, Cy5-chicken) and a matrix control (matrix, Cy5-anti-AFB<sub>1</sub>, Cy5-chicken), were run as part of the assay to determine if the matrix alone inhibited antibody binding to the biotinylated AFB<sub>1</sub>-patterned regions of the waveguide. In the case of

the corn products, the food matrix had no significant effect on antibody binding to the surface (cornflakes gave no consistently higher signals; cornmeal,  $P > 0.1$ ; popcorn,  $P > 0.25$ ). However, the presence of any of the nut products tested prevented the binding of both Cy5-anti-AFB<sub>1</sub> and Cy5-chicken to their specific targets, as exemplified in Fig. 3A for the peanut samples. In order to facilitate antibody binding in the nut samples, either 1% PEG-8000 or 4 mg/ml PVP was added to the buffer used to

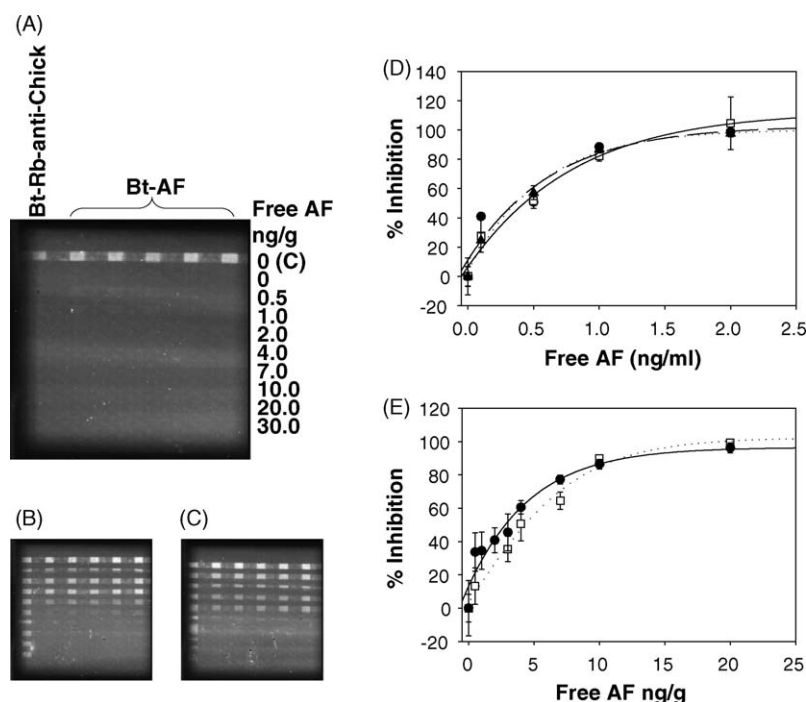


Fig. 3. Assays for AFB<sub>1</sub> in peanut samples. The CCD images show peanut samples spiked with AFB<sub>1</sub> ranging from 0 to 30 ng/g, after dilution with (A) PBSTB, (B) PBSTB + 4 mg/ml PVP and (C) PBSTB + 1% PEG-8000 (w/v). (D) Dose-response curves for AFB<sub>1</sub> (0–2.0 ng/ml) in PBSTB (dark circles), PBSTB + PVP (light squares) and PBSTB + PEG (dark triangles); no food matrix. (E) Dose-response curves for AFB<sub>1</sub> (0–20 ng/g) in PBSTB (light squares) and PBSTB + PEG (dark circles), as measured in cornflake samples. Curves were plotted as the mean % inhibition of the Cy5 anti-AFB<sub>1</sub> in the cornflake sample. Error bars are the standard deviation of the mean,  $n = 5$ .

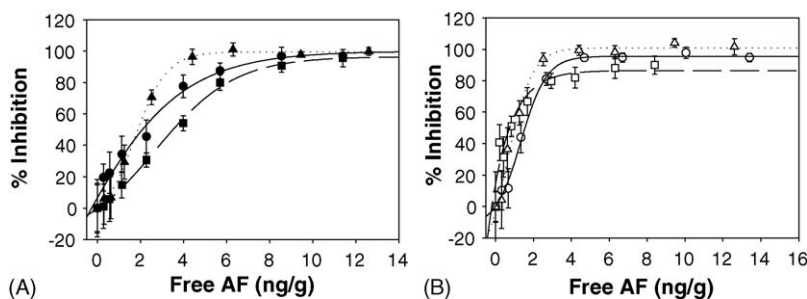


Fig. 4. Measurement of AFB<sub>1</sub> in foods. (A) Dose-response curves for AFB<sub>1</sub> (0–14 ng/g) in corn products: cornflakes (dark circles), cornmeal (dark squares) and popcorn (dark triangles). (B) Dose-response curves for AFB<sub>1</sub> (0–14 ng/g) in nut products: pecans (light circles), peanuts (light squares) and peanut butter (light triangles). Curves were plotted as the mean % inhibition of the Cy5 anti-AFB<sub>1</sub> in the sample. Error bars are the standard deviation of the mean for a minimum of two different slides each,  $n = 6$ .

dilute the extracted samples. PEG and PVP have been used previously in foodstuffs as synthetic fining agents to remove species such as polyphenols which non-specifically bind to antibodies, hence restoring antibody activity (Ogunjimi and Choudary, 1999; Visconti et al., 1999; Ngundi et al., 2005). The resulting CCD images for the peanut samples are shown in Fig. 3B and C for PEG and PVP, respectively. Clearly, addition of these compounds restores the antibody binding activity to the surface. With respect to the buffer control, PEG fully restored the antibody binding activity in the peanut sample, while the activity was restored by ~35% in the pecans and peanut butter matrices. Neither the PEG nor the PVP affected the % inhibition dose-response curve relative to buffer, as shown in Fig. 3D (in the linear range  $P > 0.25$ , as determined by ANCOVA). Likewise no significant improvement was observed in the % inhibition dose-response curve when PEG was used to dilute cornflake samples relative to PBSTB, see Fig. 3E (in the linear range  $P > 0.1$ , as determined by ANCOVA). Therefore, for these studies 1% PEG was added to the dilution buffer for the nut products only.

The dose-response curves for AFB<sub>1</sub> in the corn products (popcorn, cornflakes and cornmeal) and in the nut products (peanuts, pecans and peanut butter) are shown in Fig. 4A and B, respectively. The curves were fitted to a sigmoidal four-parameter equation and these are summarized in Table 1, along with the LODs. For the corn products, the LOD ranged from 1.5 to 5.1 ng/g depending on the food matrix, with the dynamic range occurring over a 10 ng/g change in AFB<sub>1</sub> concentration.

Both the cornflakes and cornmeal caused a significant increase in the LOD. This matrix effect is potentially due to the presence of interfering compounds other than polyphenols, as PEG did not significantly improve the LOD, Fig. 3E, and therefore must originate from another interaction. In the case of the nut products, the LODs remained low, 0.6–1.4 ng/g, and like the buffer experiments, the dynamic range of the assay was sharp, with complete inhibition occurring at concentrations  $\leq 3$  ng/g. The sharp dynamic range observed in these assays makes this particular detection system extremely useful as a qualitative screening assay where a simple yes/no response is required. With the exception of the cornmeal and cornflakes, the LODs determined were below the 2 ng/g level specified by the European Commission. These LODs are comparable to those obtained using other immunoassays (Delmulle et al., 2005, 5 ng/g pigfeed; Pal and Dhar, 2004, 0.02 ng/ml corn, wheat, cheese) including ELISAs (Lee et al., 2004, 9–12 ng/g peanuts, corn and soybeans) and the method using immunoaffinity purification coupled with LC detection developed by Urano et al. (1993, 1 ng/g in corn and nut products) or HPLC (Garcia-Villanova et al., 2004, 0.2 ng/g bee pollen). The actual biochemical analysis developed here takes 10 min to perform, which in most cases is quicker than or equivalent to other antibody-based AFB<sub>1</sub> detection methods described in the literature (Ammida et al., 2004; Lee et al., 2004; Garcia-Villanova et al., 2004; Delmulle et al., 2005). In line with previously developed food assays using the NRL array biosensor, the sample analysis is currently coupled with a 2 h extraction and 20 min antibody incubation procedure (Ngundi et al., 2005).

Table 1

Details of the sigmoidal four-parameter curves fitted to the % inhibition dose-responses obtained for AFB<sub>1</sub> in different food matrices and the resulting LODs determined as the average of the measured LODs obtained from three slides

Food matrix	Equation: $Y = \% \text{ inhibition}$ , $x = \text{AFB}_1 \text{ concentration}$	$R^2$	LOD (ng/g)
Corn products			
Popcorn	$Y = -9.72 + \{109/[1 + \exp(-(x - 1.75)/0.74)]\}$	0.999	$1.5 \pm 0.9$
Cornflakes	$Y = -152 + \{252/[1 + \exp(-(x + 1.27)/2.41)]\}$	0.993	$4.3 \pm 3.4$
Cornmeal	$Y = -22.3 + \{119/[1 + \exp(-(x - 2.71)/1.83)]\}$	0.999	$5.1 \pm 0.9$
Nut products			
Peanuts	$Y = -43.3 + \{144/[1 + \exp(-(x - 0.62)/0.66)]\}$	0.995	$1.4 \pm 1.1$
Pecans	$Y = -13.0 + \{109/[1 + \exp(-(x - 1.34)/0.68)]\}$	0.998	$1.3 \pm 0$
Peanut butter	$Y = -5400 + \{5504/[1 + \exp(-(x + 4.67)/0.62)]\}$	0.967	$0.6 \pm 0.4$

The overall time of extraction and analysis could potentially be shortened to less than an hour; however, re-optimization of the shortened method would be required to offset any potential losses in sensitivity.

#### 4. Conclusion

Rapid indirect competitive-based immunoassays have successfully been developed for the detection of AFB<sub>1</sub> in a number of corn and nut samples using the NRL array biosensor. The total time for sample testing, after a 2 h extraction, was 30 min including a pre-analysis sample/antibody incubation step; this is in line with times reported by other rapid AFB<sub>1</sub> detection systems. The assays were simple to perform and required little-to-no sample pretreatment. LODs obtained with this method are comparable to standard ELISA and more complex immunoassay methods carried out in food matrices. The main advantage of the NRL array biosensor over many of the existing technologies is its ability to measure multiple analytes in multiple samples simultaneously (Taitt et al., 2004b; Sapsford et al., 2005). This unique ability allows matrix effects to be quantitatively determined. Although multi-analyte detection was not demonstrated in this study, we have recently shown that competitive assays can be run in conjunction with standard sandwich-based immunoassays, allowing for the possibility of monitoring food samples for a variety of large and small analytes simultaneously (Sapsford et al., 2006).

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